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The Cysteine-Rich Region and Secreted Form of the Attachment G Glycoprotein of Respiratory Syncytial Virus Enhance the Cytotoxic T-Lymphocyte Response despite Lacking Major Histocompatibility Complex Class I-Restricted Epitopes

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The cytotoxic T-lymphocyte (CTL) response is important for the control of viral replication during respiratory syncytial virus (RSV) infection. The attachment glycoprotein (G) of RSV does not encode major histocompatibility complex class I-restricted epitopes in BALB/c mice (*H-2^d*). Furthermore, studies to date have described an absence of significant CTL activity directed against this protein in humans. Therefore, G previously was not considered necessary for the generation of RSV-specific CTL responses. In this study, we demonstrate that, despite lacking H-2^d-restricted epitopes, G enhances the generation of an effective CTL response against RSV. Furthermore, we show that this stimulatory effect is independent of virus titers and RSV-induced inflammation; that it is associated primarily with the secreted form of G; and that the effect depends on the cysteine-rich region of G (GCRR), a segment conserved in wild-type isolates worldwide. These findings reveal a novel function for the GCRR with potential implications for the generation of protective cellular responses and vaccine development.

Respiratory syncytial virus (RSV) is the most important viral agent causing hospitalizations related to respiratory illness in infants and young children in the United States and in the world (2, 8). About 50% of infants are infected with RSV during the first year of life, and more than 90% have been infected by the age of 2 years (2, 8). During primary infection, 30 to 70% of infants and young children develop lower respiratory tract illness (2, 8). The development of vaccines to protect children against RSV is difficult, in part because even wild-type infection does not confer long-lasting protection. No RSV vaccine has ever been licensed.

The cytotoxic T-lymphocyte (CTL) response plays an important role in the control of replication of a wide variety of viruses (34). $CD8^+$ T cells recognize major histocompatibility complex (MHC) class I molecules carrying 8- to 10-amino-acid peptides and control infection by direct destruction of infected cells or by the release of antiviral cytokines (34). In infections caused by RSV, the $CD8^+$ T cells appear to play an important role in protective immunity and recovery from infection (4, 5, 13). In addition, RSV-specific CTLs are critical for Th1 skewing of the $CD4^+$ T-cell response after vaccination (19, 37, 39). Th1 skewing is presumed to be desirable for the development of safe vaccines against RSV, because a Th2 bias of the immune response was linked to a severe form of RSV disease in recipients of a formalin-inactivated RSV vaccine subsequently exposed to wild-type virus in the 1960s (10, 14, 19, 37, 39, 41).

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The BALB/c mouse is widely used as a model for the study of RSV infection. The dominant RSV-specific CTL epitope for BALB/c mice is located at positions 82 to 90 of the antitermination factor M2-1 ($M2^{82–90}$) (9, 23, 28, 30). This H2-K^d-restricted epitope is estimated to encompass ~40% of the primary RSV-specific H-2^d-restricted CTL response (6). A subdominant CTL epitope in H-2^d mice is located in the main neutralization antigen of RSV, the fusion protein (F), positions 85 to 93 (F^{85-93}), and is responsible for <5% of the primary antiviral CTL response (7, 20). Conversely, the other neutralization antigen in RSV, the attachment protein (G), lacks H-2^d-restricted epitopes (9, 19, 26, 38). Furthermore, unlike most other RSV proteins, G has not yet been demonstrated to elicit CTL activity in humans (17, 28, 33, 49).

The RSV G protein is produced as a transmembrane form, with an N-terminal cytoplasmic tail and an N-terminal proximal hydrophobic signal anchor, and as an N terminally truncated soluble form that lacks the cytoplasmic tail and membrane anchor and is rapidly secreted (8, 27). The secreted form constituted around 20% of the total G protein synthesized in RSV-infected cells in vitro, but because of its rapid secretion it accounted for more than 80% of the G protein released in cell culture by 24 h postinfection (18). Thus, it is the major form of G presented to the host immune system early in infection. The ectodomain of the G protein includes two mucin-like segments, with divergent amino acid sequences between isolates, and a short, circumscribed central region that is highly conserved between RSV antigenic subgroups A and B (26). This conserved region includes four cysteine residues (positions 173, 176, 182, and 186) that form a cystine noose held by disulfide bonds between Cys^{173} and Cys^{186} and between Cys^{176}

and Cys^{182} (8, 26). The RSV G cysteine-rich region (GCRR) originally was presumed to play a role in receptor binding (8), but recent data have shown that it is not required for efficient infection in vitro and in mice (43, 44). However, the GCRR can modulate inflammation by inhibiting Toll-like receptor 4 (TLR4) activation and NF-κB nuclear translocation (1, 32), and a role for TLR4 in RSV clearance from infected lungs has recently been studied (16, 25). Therefore, we speculated that the GCRR might also affect the CTL response against RSV.

We describe here a novel and unexpected effect of G, one that was associated with the secreted form and was dependent on the GCRR. Despite lacking H-2^d-restricted epitopes, G is shown here to be critical for the generation of a CTL response during RSV infection. These findings are important for the current understanding of mechanisms of cell-mediated immunity against RSV and may contribute to the design of new candidate vaccines.

MATERIALS AND METHODS

Virus infection and sampling. Six- to ten-week-old female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were housed in microisolator cages in an environmentally controlled specific pathogen-free animal facility. Intranasal (i.n.) infections were performed with 5×10^5 PFU of live wild-type (wt) RSV or with the following three recombinant RSVs: (i) RSV lacking the entire G gene (ΔG); (ii) RSV expressing only the membrane but not the secreted form of the G protein (mG); or (iii) RSV lacking the GCRR ($G_{\Delta 172\text{-}187}$) (43, 44). Vaccinia virus recombinants encoding the membrane-bound and secreted forms of G (i.e., expressing the wt G gene [vvG]) or an irrelevant control gene (vvβgal) were administered i.n. at 10^4 to 10^6 PFU, as described previously (11). Plasmid cDNA encoding G or an irrelevant control gene were generously provided by Ralph A. Tripp, University of Georgia (46). DNA vaccines were administered at 100 μg/dose on days 0 and 3. All experimentation was approved by and performed according to the guidelines of the Johns Hopkins Medical Institutions and the National Institutes of Health.

Flow cytometry. Lungs were removed after sacrifice, with material from each mouse processed separately. The lungs were rinsed, minced, and digested with 3,500 Dornase U of DNase I (Calbiochem)/ml and 75 U of collagenase (Life Technologies)/ml at 37°C for 2 h and then adjusted to 0.01 M EDTA, chilled on ice, and filtered through 100-μm-pore-size nylon monofilament cloth (PGS). The cells were pelleted, resuspended, and subjected to centrifugation in Ficoll-Paque Plus solution (Amersham Pharmacia Biotech) at $400 \times g$ and 20°C for 30 min. The pulmonary mononuclear cell (PMC) interface was collected, washed twice, and resuspended in 5 ml of RPMI 1640 medium (Life Technologies) containing 10% fetal bovine serum (FBS), 100 U of penicillin/ml, and 100 μg of streptomycin sulfate/ml (3). For quantitation of RSV-specific CTL, lung PMC were isolated from mice, washed twice in phosphate-buffered saline containing 2% FBS, and stained with an optimized amount of phycoerythrin-conjugated MHC class I H-$2K^d$ tetramer complexes bearing the peptide SYIGSINNI (NIAID Tetramer Facility, Yerkes Regional Primate Research Center, Atlanta, GA), representing the immunodominant epitope of the RSV M2-1 protein (23), and fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD8α monoclonal antibody, clone 53-6.7 (BD Biosciences, San Jose, CA).

To analyze the cells that secrete gamma interferon (IFN-γ) in response to RSV-specific stimulation, PMC were resuspended in RPMI medium 1640 (Invitrogen, Carlsbad, CA) containing 10% FBS, 100 U of penicillin/ml, and 100 μg of streptomycin sulfate/ml. The cells were counted and incubated overnight with 1 μM M2-1 peptide in the presence of GolgiStop (Invitrogen). After stimulation, cells were washed twice with PBS containing 2% FBS, treated with Fc Block (BD Biosciences) to block Fc receptors, stained as described above with FITC-conjugated anti-mouse CD8α monoclonal antibody, washed twice, fixed, and permeabilized with Cytofix/Cytoperm Solution (BD Biosciences). This was followed by staining with allophycocyanin-conjugated rat anti-mouse IFN-γ antibody (clone XMG1.2; BD Biosciences). Flow cytometry analysis was performed using a FACSCalibur flow cytometer (BD Biosciences). A total of 30,000 cells were analyzed per sample.

In addition, the lytic potential of $CD8^+$ T cells was estimated by flow cytometry using a FITC-conjugated rat anti-mouse CD8α monoclonal antibody, clone 53-6.7 (BD Biosciences), and phycoerythrin-conjugated anti-granzyme B (BD Biosciences) antibody after incubation of PMC with the A-20 B-cell lymphoma line (American Type Culture Collection, Manassas, VA) loaded with the $M2^{82\text{-}90}$ peptide for 18 h.

Immunospot assay. Nitrocellulose-based 96-well microtiter plates (Millititer HA; Millipore, Bedford, MA) were coated overnight at room temperature with 10 μg of anti-IFN-γ monoclonal antibody (clone R4-6A2; BD Biosciences)/ml. Fresh PMC were incubated in the coated plates for 18 h with irradiated target A-20 B-cell lymphoma line (American Type Culture Collection) loaded with the $M2^{82\text{-}90}$ peptide. Spots corresponding to individual IFN-γ-producing cells were revealed with biotinylated anti-IFN-γ monoclonal antibody (clone XMG1.2; BD Biosciences), followed by treatment with streptavidin peroxidase and 3,3′-diaminobenzidine tetrahydrochloride dehydrate (Sigma, Saint Louis, MO).

Cytolytic activity. A standard cytolytic assay was performed using RSV-infected and uninfected cells or the $M2^{82-90}$ peptide-loaded A-20 target cells. Target cells were incubated with effector PMC in a top effector/target ratio of 50:1 in V-bottom plates (Costar/Corning Life Sciences, Acton, CA). Plates were centrifuged for 30 s at $150 \times g$ prior to a 6-h incubation at 37°C in 5% CO_2. Cells were gently pelleted, and 100 μl of supernatant fluid was transferred for the determination of released lactose dehydrogenase according to the manufacturer's instructions (Cytotoxicity Detection Kit; Roche, Indianapolis, IN). The percent specific lysis was calculated as previously described (7).

Titration of RSV in the lungs. Lungs from mice infected with 5×10^5 PFU were removed aseptically and ground in 3 ml of Hanks medium (Invitrogen). Debris was pelleted by centrifugation and samples were plated on Vero or HEp-2 cells. Monolayers were then overlaid with Opti-MEM medium (Invitrogen) with 2% fetal calf serum, 0.8% methylcellulose, glutamine, and antibiotics and incubated for 5 days. Plates were stained by the immunoperoxidase method, and the results are expressed as PFU/gram (32).

Histopathology. Lungs from mice were removed 4, 7, and 10 days after challenge, fixed overnight with 10% buffered formalin at 4°C, and embedded in paraffin. Lung sections were stained with periodic acid-Schiff reaction to examine the inflammatory infiltration. Examination was performed by two blinded observers, and the results were subsequently averaged. Briefly, to characterize the pneumonia, the vessels and bronchi were initially scored as follows: 1, free from or with few infiltrating cells; 2, shwoing focal aggregates of infiltrating cells or the structure cuffed by one definite layer of infiltrating cells; or 3, having two or more definite layers of infiltrating cells with or without focal aggregates. Subsequently, the histopathology was categorized as mild (>60% with score = 1; 0% with score = 3), moderate (>30% score = 2; <20% score = 3), or severe (>20% score = 3).

Statistical analysis. Data were analyzed with statistical software (Statview; SAS Institute Inc, Cary, NC). Comparisons were made by using the Mann-Whitney U test where appropriate.

RESULTS

The G protein is critical for the RSV-specific CTL response. To determine the role of G in the RSV-specific CTL response, we compared the numbers of RSV-specific CTL in mice after i.n. infection with wt RSV, the recombinant RSV that lacks the entire G gene (ΔG), and the recombinant RSV that expresses only the membrane but not the secreted form of the G protein (mG). On various days postinfection, PMC were isolated. The yields of cells were similar for all viruses. CTLs were analyzed by three different methods. First, the number of RSV-specific $CD8^+$ T cells were determined by staining PMC with anti-CD8 antibody and a tetramer specific to the RSV $M2^{82\text{-}90}$ immunodominant epitope (Fig. 1a). Second, to quantitate the T cells secreting IFN-γ in response to specific stimulation, PMC were stimulated with the RSV $M2^{82\text{-}90}$ CTL immunodominant peptide, stained for CD8 and IFN-γ, and analyzed by flow cytometry (Fig. 1b). Third, the number of cells secreting IFN-γ in response to specific stimulation was determined by immunospot assay (Fig. 1c). The virus-specific $CD8^+$ T-cell response induced by wt RSV was detectable by flow cytometry at 5 days and peaked 9 days after infection (Fig. 1a and b; $P < 0.01$ versus mG and ΔG). The kinetics of the CTL response elicited by the two viruses lacking one or both forms of G were delayed.

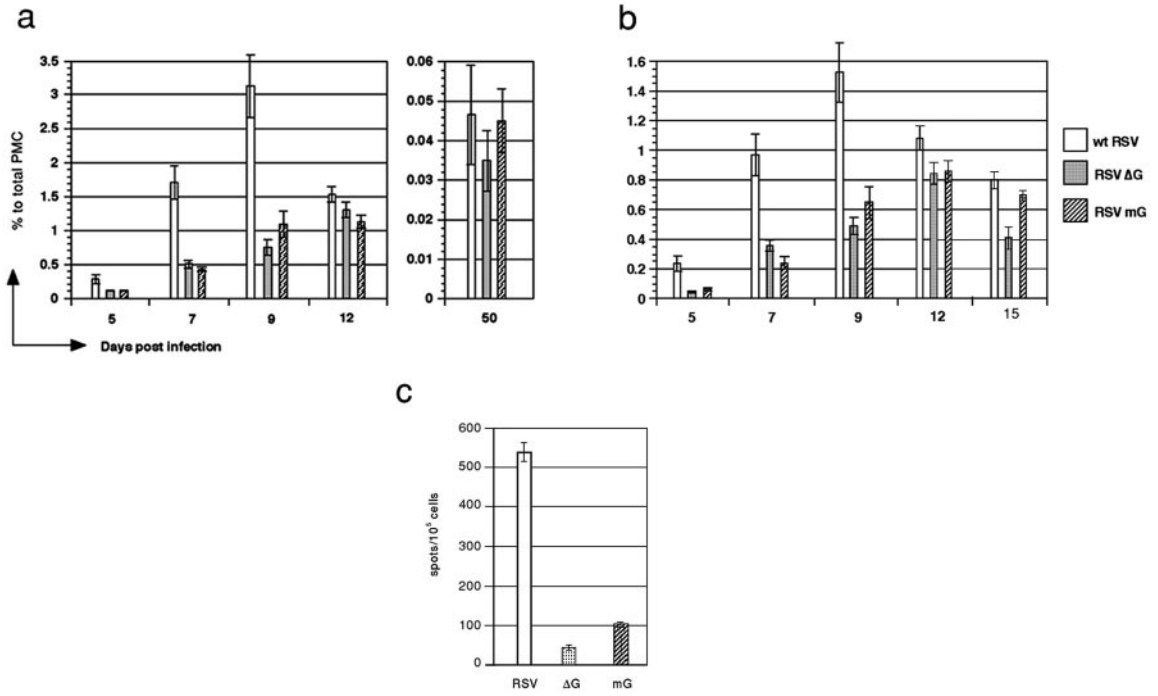

FIG. 1. Quantification of RSV and M2-specific $CD8^+$ T cells during infection with RSV or recombinant G-deficient viruses. (a) PMC were isolated at the indicated time points postinfection, stained with anti-CD8 antibodies and the M2 tetramer, and analyzed by flow cytometry. (b) PMC were stimulated with the $M2^{82-90}$ peptide, stained for CD8 and IFN-γ, and analyzed by flow cytometry. (c) PMC isolated at the peak of the CTL response on day 9 were stimulated with the $M2^{82-90}$ peptide-loaded A-20 target cells, stained for IFN-γ, and analyzed by an immunospot assay. Bars: □, RSV; ▨, ΔG; ▨, mG. The results are means ± the standard errors of the mean (SEM) and are representative of two to three independent experiments.

Interestingly, the number of RSV-specific CTL induced by ΔG and mG was lower than the response elicited by wt RSV at all time points. All three primary responses decreased by day 15 after inoculation (Fig. 1b). This observation suggests that the secreted form of the G protein is necessary for eliciting an effective RSV-specific CTL response. As previously described for wt RSV (6), on day 50, the responses evaluated by tetramer staining decreased to very low levels for all viruses (Fig. 1a) and were not detectable by IFN-γ staining (not shown).

The G protein is critical for RSV-specific cytolytic activity. Next, we determined whether the differences in CTL numbers evaluated by tetramer and IFN-γ staining were associated with differences in cytolytic activity. The levels of cytolysis were evaluated ex vivo by measuring the release of lactate dehydrogenase from RSV-infected and $M2^{82-90}$-specific target cells exposed to the PMC of infected mice (Table 1). High-level cytolytic activity was detected 7 days after infection, and the response decreased by day 12 in all groups. As observed with IFN-γ production, cytolysis was greater in mice infected with wt RSV than in animals infected with ΔG or mG ($P < 0.01$).

Coadministration of RSV G during infection can enhance the CTL response. We then examined whether the simultaneous administration of a vector expressing RSV G protein (expressing both the membrane-bound and the secreted forms) with the mG virus (expressing only the membrane-bound form) could reestablish a RSV-specific CTL response of similar magnitude as that elicited by wt RSV infection. For this purpose, we infected mice intranasally with wt RSV, with mG alone, or with mG with a recombinant vaccinia virus expressing the RSV G gene (vvG) or an irrelevant control gene (vvβgal). Interestingly, $M2^{82-90}$-specific CTL activity measured in PMC isolated 7 days postinfection was restored by the coadministration of vvG with mG to levels similar to those detected after infection with wt RSV (Fig. 2A). Conversely, the coadministration of vvβgal and mG resulted in low responses, similar to those observed after infection with mG alone. These findings support a role for the secreted form of the G protein in promoting RSV-specific CTL responses during infection.

Next, we examined whether incremental addition of G to wt RSV infection could further increase the RSV-specific CTL response in a dose-dependent manner (Fig. 2B). Therefore, we inoculated mice with wt RSV and incremental doses of vvG or

TABLE 1. RSV-specific cytolytic responses after infection with RSV or recombinant G-deficient viruses[a]

Virus	Mean cytolytic activity ± SEM			
	RSV		$M2^{82-90}$	
	Day 7	Day 12	Day 7	Day 12
RSV	75.4 ± 4	21.8 ± 3	37.5 ± 6	24 ± 6
ΔG	57.8 ± 4	12.7 ± 3	18.2 ± 4	4.2 ± 3
mG	54.5 ± 6	15.4 ± 6	22 ± 3	14.6 ± 4

[a] PMC were stimulated with RSV-infected or $M2^{82-90}$-peptide-loaded A-20 target cells for 6 h., and the cytolytic activity was tested using the lactate dehydrogenase release assay with a 50:1 effector/target ratio.

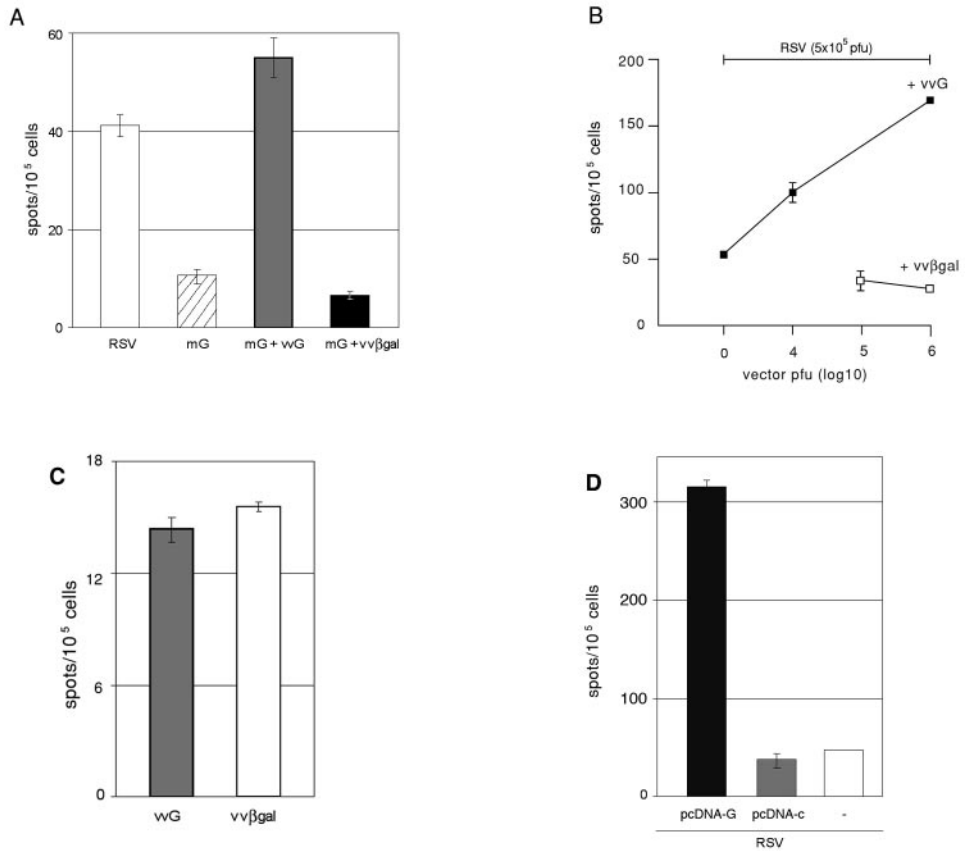

FIG. 2. Quantification of RSV-specific $CD8^+$ T cells after infection with wt RSV, mG, or coinfection with vectors encoding protein G. (A and B) PMC were isolated on day 7 after infection, stimulated with $M2^{82-90}$ peptide-loaded A-20 target cells, stained for IFN-γ, and quantitated by using an immunospot assay. In panel A, the indicated RSVs were administered i.n. at a dose of 5×10^5 PFU, and the indicated vaccinia virus recombinant was administered i.n. at a dose of 10^5 PFU. Results are expressed as means ± the SEM and are representative of two independent experiments. In panel B, wt RSV and each recombinant vaccinia virus were administered i.n. at the indicated dose (vector PFU). (C) PMC were isolated on day 7 after vvG or vvβgal infection, stimulated with vaccinia virus-infected A-20 target cells, stained for IFN-γ, and quantitated by using an immunospot assay. (D) RSV was administered i.n. at a dose of 5×10^5 PFU, and the indicated pcDNA was administered i.n. at 100 μg/dose on days 0 and 3. The results are expressed as means ± the SEM.

vvβgal. Interestingly, the addition of vvG (at all doses tested) increased the RSV-specific CTL response. This enhancement was dose dependent; infection with 10^6 PFU of vvG resulted in an increase of the enhancing effect by 70% compared to 10^4 PFU of vvG and suggested that physiologic RSV-specific CTL responses may be further increased by the addition of protein G.

To examine the specificity of the RSV G protein effect and its potential role in CTL responses against other viruses, we quantitated the vaccinia virus-specific CTL response in BALB/c mice after i.n. inoculation with vvG and vvβgal (Fig. 2C). Interestingly, the vaccinia virus-specific CTL responses elicited by both viruses were of similar magnitude, demonstrating that the positive modulatory effect of G is not universal.

Finally, to determine whether the positive modulatory effect on the RSV-specific CTL response elicited by G was also induced when a cDNA vector was used, we inoculated mice with wt RSV and a pcDNA encoding G (pcDNA-G) or a pcDNA encoding an irrelevant control gene (pcDNA-c) (Fig. 2D). Interestingly, the $M2^{82-90}$-specific response after coadministration of wt RSV plus pcDNA-G exceeded the response elicited by wt RSV or wt RSV plus pcDNA-c by >8-fold ($P < 0.01$).

The differences in CTL activity are not explained by differences in the pulmonary replication of wt and recombinant viruses or differences in the inflammatory response. The magnitude of the CTL response is often determined by the virus titer during infection. Therefore, to examine whether the differences in CTL response were associated with differences in replication, we compared the kinetics of virus titers in lungs after infection with wt RSV or the recombinant viruses lacking one or both forms of G (Fig. 3A). Even though wt RSV and mG elicited significantly different CTL responses (wt RSV> mG; see Fig. 1 and 2), both viruses replicated to similar titers, while replication of ΔG was further reduced and was detectable only in two of five infected animals. However, the CTL response elicited by ΔG was similar to that of the mG recipients. These findings demonstrate that the differences observed in the magnitude and lytic capacity of CTL responses elicited by the different RSV constructs cannot be explained by differences in their respective pulmonary titers.

In addition, G-deficient RSV recombinants promote signif-

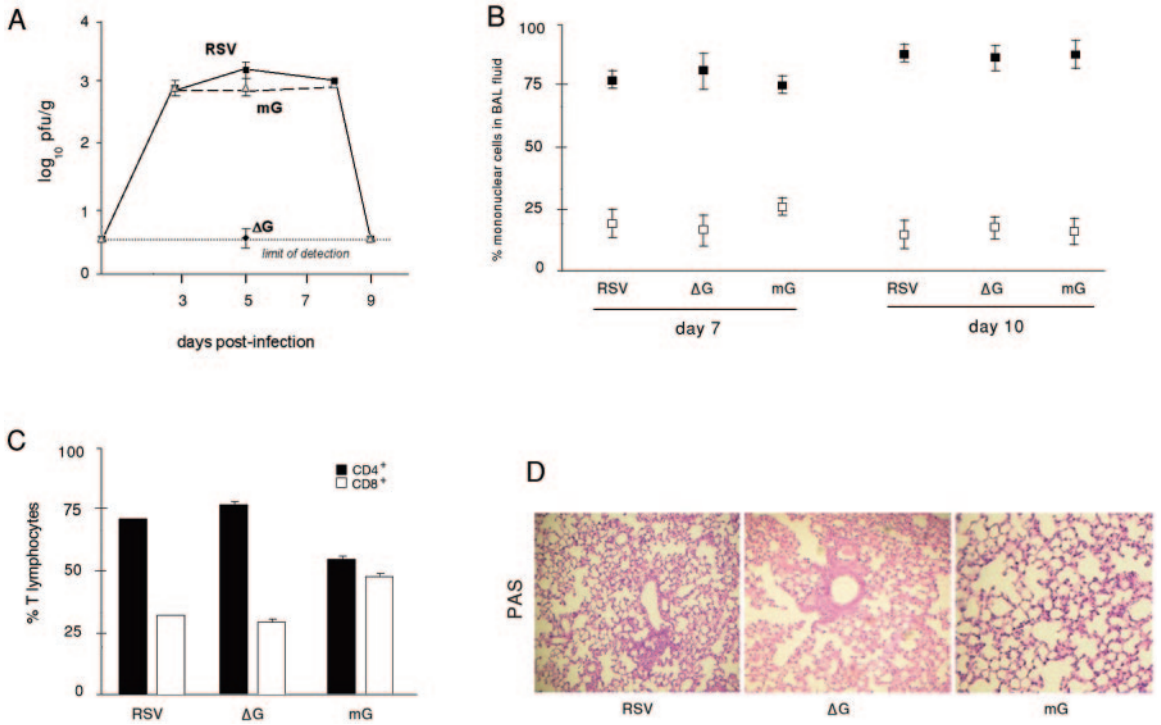

FIG. 3. Viral lung titers, differential cell counts in BAL fluids, and pulmonary histopathology. (A) Virus titers in lungs after infection with 5×10^5 PFU of wt RSV or the recombinant viruses lacking one or both forms of G. ■, RSV; △, mG; ◆, ΔG. The results are expressed as means ± the SEM (five mice/group). (B) Percent lymphocytes and monocytes in mononuclear cells obtained from BAL fluids in mice 7 and 10 days after infection with RSV or G-deficient viruses. □, Monocytes/macrophages; ■, lymphocytes. The results are expressed as means ± the SEM. (C) Percent $CD4^+$ and $CD8^+$ pulmonary T lymphocytes obtained from BAL fluids in mice 7 days after infection with RSV or G-deficient viruses. (D) Pulmonary histopathology in mice during peak inflammation at 7 days after infection with RSV or G-deficient viruses (periodic acid-Schiff staining, ×10 magnification).

icant innate inflammation in the lungs of mice early (24 h) after inoculation (32). Therefore, a possible trivial explanation for the poor CTL response detected in mice infected by these viruses might have been a relative excess of pulmonary macrophages over lymphocytes in these samples compared to those obtained from wt RSV recipients. This relative excess of macrophages could decrease the proportion of lymphocytes in the total cells selected for the assays. However, the percent lymphocytes in PMC isolated from the bronchoalveolar lavage (BAL) fluids of mice 7 and 10 days after infection with RSV, mG, or ΔG were not different and ranged between 70 and 85% of cells (Fig. 3B). Indeed, the percentages and absolute numbers of the $CD4^+$ T cells in BAL fluids of all groups of mice were similar and exceeded the percentages and absolute numbers of pulmonary $CD8^+$ T cells on days 7 (Fig. 3C) and 10 (data not shown). These findings confirm that the observed effect cannot be attributed to a relative excess of $CD4^+$ T cells in the detriment of $CD8^+$ T cells in the lungs of mice immunized with ΔG or mG. In addition, comparison of pulmonary histopathology on days 4, 7, and 10 postinfection showed that, due to the relatively low dose of the inoculum (5×10^5 PFU), only mild perivascular and peribronchiolar granulocytic and mononuclear cellular infiltration with mild alveolitis was present in all groups as peak inflammation on day 7 after infection (using criteria described in Materials and Methods) (Fig. 3D).

The conserved GCRR is necessary to elicit RSV-specific cytotoxicity. Recently, the conserved GCRR was shown to have immune modulatory properties during RSV infection (32). Therefore, we investigated whether the GCRR could affect CTL activity. To address this question, we compared the $M2^{82\text{-}90}$-specific CTL response elicited by wt RSV and the recombinant RSV lacking the GCRR ($G\Delta_{172\text{-}187}$). For this purpose, we used an immunospot assay to quantitate the number of IFN-γ-positive cells at the peak of the CTL response (Fig. 4A). Even though $G\Delta_{172\text{-}187}$ virus titers in the lungs were similar to those of wt RSV (Fig. 4C), the CTL response in PMC from mice infected with $G\Delta_{172\text{-}187}$ was significantly lower than the response observed 10 days after wt RSV infection (Fig. 4A; $P < 0.01$). As previously described for other recombinant viruses, the percentages of $CD4^+$ and $CD8^+$ T cells in BAL fluids from $G\Delta_{172\text{-}187}$-immunized mice did not differ from those observed in wt RSV-infected animals (not shown).

Finally, we examined the CTL lytic activity on a per-cell basis by measuring $CD8^+$ T-cell production of granzyme B after infection of BALB/c mice with wt RSV or $G\Delta_{172\text{-}187}$ (Fig. 4B). Again, infection with wt RSV resulted in higher levels of granzyme B production than with the recombinant virus lacking the GCRR on days 7 and 12 after infection. These observations strongly suggest that the GCRR is critical for the production of an effective CTL response against RSV.

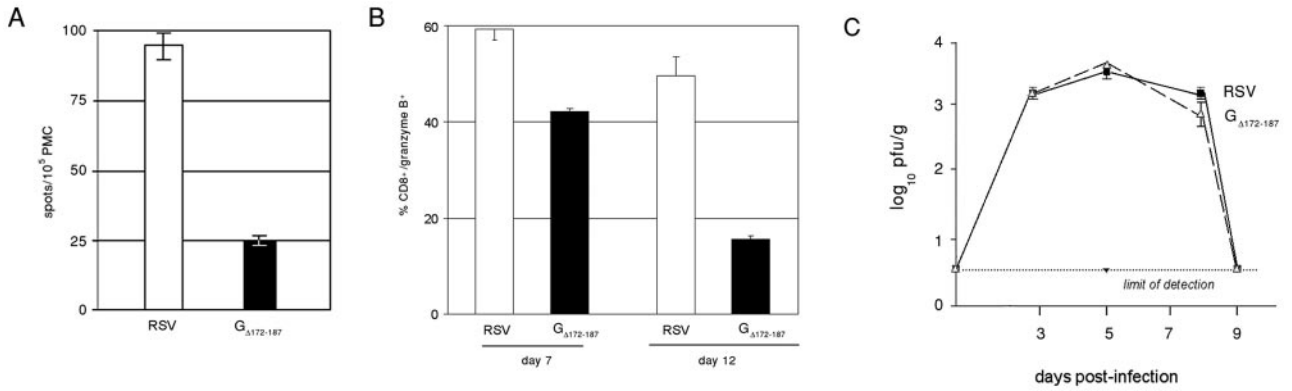

FIG. 4. Quantification of the RSV-specific $CD8^+$ T cells after infection with wt RSV or the $G\Delta_{172\text{-}187}$ virus. (A) PMC were isolated from infected mice on day 10 postinfection and stimulated with the $M2^{82\text{-}90}$ peptide-loaded A-20 target cells, and the numbers of cells producing IFN-γ were determined by an immunospot assay. Bars: □, wt RSV; ■, $G\Delta_{172\text{-}187}$ virus. The results are expressed as means ± the SEM and are representative of three independent experiments. (B) Percent expression of granzyme B by $CD8^+$ T cells isolated from infected mice on days 7 and 12 after infection with wt RSV or the $G\Delta_{172\text{-}187}$ virus. After isolation, PMC were stimulated with $M2^{82\text{-}90}$ peptide-loaded A-20 target cells. Bars: □, wt RSV; ■, $G\Delta_{172\text{-}187}$ virus. The results are expressed as means ± the SEM and are representative of two independent experiments. (C) Titers of the viruses in lungs after infection of mice with wt RSV (■) or the $G\Delta_{172\text{-}187}$ virus (△). The results are expressed as means ± the SEM (four to five mice/group).

DISCUSSION

This study describes a novel role for the RSV G protein and its GCRR in RSV immunobiology. Despite lacking $H\text{-}2^d$-restricted epitopes, G is necessary for the development of an effective RSV-specific CTL response during primary infection. This pro-CTL effect is associated, at least in part, with a widely conserved central segment of the protein, the GCRR. Furthermore, infection with mG, a recombinant virus that does not express the secreted form of G (including its GCRR), resulted in a significantly reduced CTL response. Therefore, these findings suggest that secretion of G with its GCRR is critical for the generation of RSV-specific cytotoxicity.

The CTL response is important for the control of RSV replication in the respiratory tract. Depletion of $CD8^+$ T cells in mice results in elevated virus titers 7 days after infection and delayed pulmonary clearance (13). Also, immunization of mice with a recombinant vaccinia virus expressing the RSV immunodominant $H\text{-}2^d$-restricted epitope encoded in $M2^{82\text{-}90}$ conferred protection against RSV challenge (9). However, this protective effect mediated by CTL waned within several weeks, and CTL did not appear to make a significant contribution to long-term protection associated with prior infection or vaccination. Although CTL might not make a significant contribution to long-term immunity as a direct antiviral effector, CTL do appear to play a critical role in regulating the immune response through the secretion of factors, notably IFN-γ (31).

The GCRR pro-CTL effect modifies a long-standing paradigm in RSV immunology: the idea that G is not necessary for the generation of a CTL response against RSV. Our data suggest that, despite the apparent absence of a significant CTL response to RSV G in mice or humans, the protein plays a critical role in the induction of RSV-specific CTL. This conclusion is consistent with a recent report showing an enhancement of cellular responses associated with expression of a G segment located at amino acids 125 to 225 with a chimeric CTL epitope and the DsbA carrier protein, although that effect was hypothesized to result from a Th epitope located between amino acids 181 and 203 (12).

The observation that supplementation with RSV G can enhance the specific CTL response in a dose-dependent manner beyond its natural level has interesting implications for vaccine design. The incorporation of additional copies of the G gene in recombinant viruses and/or relocation of the gene upstream in the viral genome to enhance its transcription and/or increasing the relative expression of the secreted form of G are strategies that may improve the cellular response against RSV. However, the theoretical benefit of enhancing the RSV-specific CTL response should be carefully weighed against other potential modulatory properties of the GCRR (32) and other regions of G (19, 36, 38).

The mechanism of the pro-CTL effect of the GCRR is intriguing. We are not aware of any other viral segments that, lacking MHC class I-restricted or Th epitopes, promote virus-specific cytotoxicity. While this effect presumably enhances viral clearance and thus would have a negative effect on virus replication, there may be a trade-off with beneficial effects that also are mediated by the GCRR. For instance, the GCRR downregulates the production of inflammatory cytokines mediated by TLR4 early after infection (32). Modulating TLR4 may play a role in the pathogenesis of RSV, an idea that is consistent with the observation that infants with loss-of-function single nucleotide polymorphisms in TLR4 are epidemiologically associated with an increased severity of illness and a decreased oxygen saturation in response to RSV infection (40). However, this TLR4 modulation also affects the production of interleukin-10 (IL-10), a cytokine involved in the downregulation of CTL responses in other models (24, 32). Therefore, the anti-inflammatory effect of the GCRR on TLR4 may be balanced by a surge in the RSV-specific CTL response resulting from decreased IL-10 production. A second potential explanation for the observed effect of the GCRR on CTL induction may be associated with the fractalkine motif encompassed in

the GCRR between amino acids 182 and 186, which is also disrupted in $G\Delta_{172\text{-}187}$ (45). Fractalkine may enhance CTL activity through chemoattraction and the activation of dendritic cells (15, 29). A third potential explanation for the observed effect is a RSV G-specific stimulation of $CD4^+$ T cells leading to secondary expansion of the $CD8^+$ T-cell subset supported by enhanced $CD4^+$ T-cell help. A single immunodominant I-E^d epitope spanning RSV G amino acids 183 to 198 and largely restricted to a subset of $CD4^+$ T cells expressing Vβ14 in the T-cell receptor has been described for BALB/c mice immunized with vvG (21, 22, 37, 39, 42, 47, 48) and partially overlaps the GCRR (32). However, the response to $G_{183\text{-}198}$ by $CD4^+$ T cells is genetically restricted, whereas the effects of the GCRR extend to other murine strains, including a similar positive modulatory effect against the recently described H-2^b-restricted epitope in the matrix protein of RSV (35; data not shown). Finally, the anti-inflammatory effect of the GCRR may affect the number of antigen-presenting cells (3), or macrophage activation through TLR4 (as observed with mG, ΔG, and $G\Delta_{172\text{-}187}$) may contribute to suppress T-cell activation (32). An important clue to a possible mechanism is that the pro-CTL effect of G observed for anti-RSV responses does not necessarily translate to cytotoxic responses directed against other viruses, in this case vaccinia virus. Thus, the differential effect on the CTL response against RSV versus vaccinia virus elicited by RSV G suggests that a direct, nonspecific, chemoattractive effect is less likely to explain this pro-CTL phenomenon. Careful evaluation in additional models may determine whether G can be used as an adjuvant in specific situations.

In summary, we describe here a novel and unexpected role for the cysteine-rich region of RSV G. This positive modulatory effect on CTL function may be important for RSV vaccine design. Furthermore, whether the GCRR can be used to elicit a broader beneficial effect on protective CTL responses against other illnesses should be investigated.

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